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An Autoradiographic Study of Cell Kinetics in Epidermis of the Toad *Bufo bufo bufo* (L)

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Under normal conditions, the toad *Bufo bufo bufo* molts about once a week. It was considered of interest to study whether the rhythmic pattern of cell loss through shedding of a stratum corneum is correlated with a rhythmic production of cells in stratum germinativum. An autoradiographic investigation of cell kinetics in toad skin using [^{3}H]-thymidine and [^{14}C]-thymidine as precursors revealed that cell production in stratum germinativum is a continuous process, and the cell production rate is much higher than is required for replacement of the cells lost with each molt. The parameters measured, i.e., % labeled cells, length of cell cycle, and S-phase, as well as migration of labeled cells from stratum germinativum toward stratum corneum support the assumption of an inhomogeneous cell population in stratum germinativum. A model proposed by Potten for the cellular organization of mouse epithelium provides a useful basis for the interpretation of the results obtained in the present study.

Adult toads, *Bufo bufo bufo*, molt about once a week. During the molting process, the 1-cell-layer-thick stratum corneum is shed, and the replacement layer becomes a new stratum corneum. As a rule, the entire slough is removed by the animals with their front legs when it has ruptured on the back; they then eat it. This process takes only a few minutes.

In a previous paper, Jørgensen and Levi [1] have shown that the rhythmic pattern of cell loss from the toad epidermis is correlated with a continuous production of cells within the stratum germinativum. Hence, the toad epidermis represents a system in which the outflux of cells per unit time can be measured with considerable accuracy by counting the number of cells per unit area shed with each slough. Also the cell production rate in stratum germinativum can be determined fairly accurately from the labeling index after injection of tritiated thymidine.

The present paper deals with a more detailed analysis of cell kinetics in the toad epidermis, i.e., measurements of labeling index, cell cycle time, duration of the S-phase, migration of cells from stratum germinativum toward stratum corneum, as well as estimates of cell fluxes. The data obtained are interpreted in terms of a simplified model proposed by Potten [2].

MATERIALS AND METHODS

Male toads *Bufo bufo bufo* caught either in the spring or in the fall were used throughout. They were kept in outdoor ponds, but at least 4 weeks prior to the start of an experiment, animals were transferred to the laboratory and kept in a temperature-regulated room at 20°C. They had access to water and were fed mealworms about twice a week. Their molting cycles were recorded using lipstick marking of the dorsal skin as described by Bendsen [3].

[^{3}H]-Thymidine (20 Ci/mmol) and ^{14}C-labeled thymidine (40–60 mCi/mmol) were purchased from New England Nuclear Corp. The animals were injected into the lymph sac with a constant dose of 2.5 μCi/g body weight, and the first injection was always given at 11 AM. The animals were then placed individually into small plastic containers on moist filter paper.

One finger was removed whenever a skin sample was needed for study. This sample was fixed in 4% neutral formalin. After incision on the ventral side, the bone was removed under the dissection microscope, the tissue was dehydrated and embedded in tissuemat. Sections were cut at 6 μm.

The autoradiographic procedure was strictly standardized. Ilford K 5 emulsion was diluted 1 part emulsion plus 2 parts distilled water at 43°C and filtered through gauze to remove air bubbles. After processing of the plates, the tissue was stained with hematoxylin and eosin and mounted in Depex.

Autoradiograms were analyzed by visual cell and grain counting under oil immersion. In most cases, 500–1000 cells from each tissue sample were evaluated. They were grouped as zero grain nuclei, nuclei with 1–3 grains, 4–14 grains, 15–40 grains, and >40 grains/nucleus. Moreover, for detailed analyses of grain distributions, e.g., as a function of time after injection of the precursor, the number of grains over each cell nucleus was registered.

Background was determined by counting the number of grains/unit area in the emulsion at a distance of several hundred microns from the tissue section, and also by determining the distribution of grains over cell nuclei in an inactive control section coated and processed together with "active" sections. The probability for a given number of background grains over a well-defined area (nucleus) can then be calculated.

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Abbreviations:
LI: labeling index
str: stratum

A more detailed account of the grain count evaluations is given in the Appendix.

RESULTS

The Size of Cell Compartments in Toad Epidermis

The toad skin consists of an outermost stratum corneum, an underlying replacement layer, an intermediate zone, 2–3 cell layers thick, and a stratum (str) germinativum where cell proliferation takes place. The ratio of number of str germinativum cells : str spinosum cells : str corneum cells was found from cell countings on 6-μm paraffin sections of the thumb's skin to be of the order of 30:60:10. Similar investigations have been carried out by Budtz [4], who used 1- or 2-μm epon sections of abdominal skin. He arrived at results not too different from those obtained on thick paraffin sections: str germinativum plus str spinosum = 10 · str corneum. Budtz also discussed the results obtained when using different approaches and modes of calculation.

Labeling Index 2 Hours after Injection of the Precursor

The percentage of str germinativum cells labeled 2 hr after injection of [^{3}H]-thymidine at 11 AM was determined on 35 toads, animals in the molting phase not included (Table I).

The labeling index (LI) is listed in the last column as compared to the number of labeled cells to be expected (column 3) if the LI were the average of 7.9%. A χ^2-value is calculated which is significant at the 0.1% level.

Grain Distribution for Background and for Labeled Cell Nuclei 2 Hours after Injection of the Precursor

As mentioned above, the number of grains over the labeled nuclei has been evaluated in two ways. With a background of 0.2 grains/100 μm^2, the probability of 3 grains over a cell in an emulsion covering an inactive tissue section is very small (~0.01%) [5]. "Background" or spurious grains present in the emulsion should by definition be expected to follow a Poisson distribution.

The grain distribution over cells with more than 4 grains/cell, however, shows a marked discrepancy from Poisson (Table II), especially due to a preponderance of cells with a few, but more than 4, grains/cell.

TABLE I. *Percentage of labeled stratum germinativum cells 2 hours after injection of the precursor (35 toads)**

No. of cells			L.I. %	No. of cells			L.I. %
Counted	Labeled			Counted	Labeled		
	Obs	Exp			Obs	Exp	
1138	172	89.6	15.1	755	51	59.4	6.8
605	69	47.6	11.4	773	51	60.8	6.6
696	76	54.8	10.9	123	8	9.7	6.5
3873	417	304.8	10.8	128	8	10.1	6.3
142	14	11.2	9.9	661	40	52.0	6.1
1132	111	89.1	9.8	1764	101	138.8	5.7
634	59	49.9	9.3	931	53	73.3	5.7
2379	213	187.2	9.0	781	40	61.5	5.1
677	61	53.3	9.0	699	35	55.0	5.0
1791	154	141.0	8.6	123	6	9.7	4.9
448	38	35.3	8.5	576	27	45.3	4.7
1628	137	128.1	8.4	842	39	66.3	4.6
572	48	45.0	8.4	782	35	61.6	4.5
681	56	53.6	8.2	794	32	62.5	4.0
1533	126	120.7	8.2	724	27	57.0	3.7
835	66	65.7	7.9	641	23	50.5	3.6
2188	172	172.2	7.9	832	30	65.5	3.6
1016	74	80.0	7.3				
			Total: 33,897	2,668	2,668.1		7.9

* According to the null hypothesis, the expected number of labeled cells is the number counted × 0.079. Therefore, the sum must be the same for both columns (*Obs* and *Exp*). The differences between the individual observed and expected values are significant, and it follows that the observed variations in the L.I. are not due to random fluctuations only.

TABLE II. *Distribution of cells according to number of grains/cell in stratum germinativum and stratum spinosum at different times after injection of the precursor*

No. of grains/cell	Stratum germinativum	Stratum spinosum	
	2–4 hr	2 hr–6 days	10–39 days
4	7	11	16
5	4	3	5
6	3	4	6
7	2	0	4
8	2	5	6
9	2	3	3
10	0	1	0
11–15	5	4	5
16–20	12	3	2
21–25	7	1	1
26–30	9	4	0
31–40	17	2	0
41–	37	13	0
Total	107	54	48
No. of toads	9	6	4

It is seen that, at all times, heavily labeled cells occur more frequently in str germinativum than in str spinosum.

Percent Labeled Cells and Grain Distribution as a Function of Time

In order to study the fate of str germinativum cells labeled 2 hr after injection of [^{3}H]-thymidine, skin samples were taken at intervals after injection of the precursor. Some of the labeled cells are expected to divide, while others are bound to leave str germinativum.

The observations depicted in Fig 1 indicate a decrease in the % of labeled cells in str germinativum within a few hours after injection of the precursor followed by an increase seen over a period of a week, thus indicating that the rate of disappearance of labeled cells is slower than that of cell division in this cell layer.

A more detailed picture is obtained when the grain distribution over labeled nuclei is studied as a function of time. This analysis is illustrated in Fig 2 which shows the mean number of grains/cell as a function of time on a semilog scale. As is to be expected, the curves approach asymptotically the limit of 4 grains/cell—by definition a "labeled cell." The grain halving time, T, found from the tangent to these curves at time zero is of the order of 4 days. This can be taken as a measure of the cell cycle time.

Duration of the S-Phase

The length of the S-phase has been determined using the double-labeling technique described by Schultze and coworkers [6, 7]. The animals were injected with [^{3}H]-thymidine at 11 AM and with [^{14}C]-thymidine 1 hr later. The results obtained on 10 animals are presented in Table III. The mean value for S is found to be 7 ± 2 hr. The variability of this value is of the same order as that of the labeling index.

Migration of Cells from Str Germinativum to Str Spinosum

For a period of at least 3 days after injection of the precursor, labeled cell nuclei are rarely seen outside str germinativum. Five days after the injection, they appear in str spinosum, and this migration has been followed over a period of up to 39 days. An analysis of grain distribution over the labeled str spinosum cells as a function of time is shown in Table II.

For this analysis, the results obtained at different time intervals had to be pooled. When comparing the grain distribution

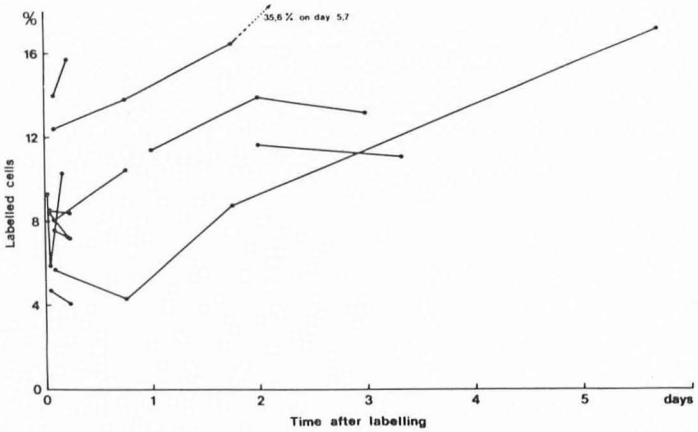

FIG 1. Percentage of labeled cells in stratum germinativum as function of time after injection of the DNA-precursor. Toads with more than 1 determination in the course of 6 days. Results from the same toad connected by *lines*. Comment: As is to be expected, in the course of the first hours after injection, the percentage of labeled cells decreases due to the influx of unlabeled cells. Thereafter, an increase will occur, now due to division of labeled cells. In some animals the increase is seen to prevail until day 5 or 6.

with that in str germinativum 2 hr after injection, it is obvious that, at all times, heavily labeled cells occur more frequently in str germinativum than in str spinosum.

Cell Flux Measurements

A different approach to a determination of S (length of S-phase) and T (cycle time) is a study of cell flux into the S-phase. Two animals were injected 4 times at intervals of 3 hr (¼ the usual dose each time) and samples were taken 2 hr after each injection. In a later experiment each of 3 animals received a total of 9 injections at 8-hr intervals, i.e., over a period of 64 hr with a total dose 4 times the normal. Samples were taken 2 hr after the first, fourth, seventh, and ninth injection, and about 500 cells were counted in each case.

From the first experiment, we can estimate the length of the S-phase to be 12 hr and 7 hr, respectively, in satisfactory agreement with previous results. The fluxes, i.e., the percentage of cells entering the S-phase per hour, turn out to be 0.64 and 0.73, respectively.

One of the animals in the second experiment was in the molting phase at the time of the first injection and shows an extraordinarily low LI of 0.6%. The correlation between cell cycle parameters and molting phase will be dealt with in a later publication. The length of the S-phases calculated from t = 2 hr till t = 26 hr are 14 and 10 hr, respectively. The fluxes here are 0.44 and 0.52.

The percentage of labeled cells in str germinativum after 9 injections in the course of 64 hr was 28.5%, in contrast to an expected value of 71% (LI = 7.9% × number of injections) plus a contribution from cells that divided in the course of the 64-hr period. A satisfactory explanation of this observation is lacking; however, similar findings were discussed by Potten and coworkers [8, 9].

DISCUSSION

It appears from Table I that the LI is not the same in all animals: 95% of the toads studied should have a LI between 4.2% and 11.6% irrespective of site (*cf.* [1]) due to both biologic and technical variations. On an average, about 8% of the cells in str germinativum are in the S-phase at any time. The significance of this observation becomes clear if the labeling index is viewed in relationship to the relative number of cells present in different layers of the toad epidermis. As mentioned above, about 30% of all cells are located in str germinativum. Hence 8% of these 30%, that is 2.4% of the total cell population, are in the S-phase at any time. Since the length of the S-phase is of the order of 7 hr and the intermolt period is of the order of 7 days, it follows that the cell population increases by 7 days/7 hr · 2.4% = 57.6% between two molts, while only 10% of the cells are lost with each molt.

From these figures it must be concluded that the production rate of cells in str germinativum exceeds the rate of cell loss through molting by a factor of about 6.

An attempt to follow labeled str germinativum cells on their way toward str corneum has revealed that (1) the number of labeled cells visible in str spinosum in the course of up to 4 weeks after administration of the precursor is very small, (2) the cells observed in that region predominantly carry a weak label, and (3) the percentage of cells with only very few grains increases markedly with time. This seems to indicate that labeled cells remain in str germinativum for a period of time long enough to undergo at least one division.

The few labeled cells moving out of str germinativum are rarely observed in the proximity of the replacement layer, which indicates either that the label is lost, e.g., in the process of maturation, or some of the cells disintegrate. Labeled material lost by disintegrating cells may give rise to a diffuse, weak autoradiogram over the tissue (an increased background). Obviously, a weak primary DNA-labeling cannot be distinguished from a slight redistribution of radioactive material occurring at a later time.

The length of the cell cycle determined from the change in grain distribution with time, and from the grain halving time, was found to be about 100 hr or 4 days. The length of the S-phase was of the order of 7 ± 2 hr. Since the S-phase determination is based on the ratio

$$\frac{\text{number of } (^{14}\text{C-labeled cells + double-labeled cells})}{\text{number of cells labeled with } ^{3}\text{H only}}$$

the difficulty in distinguishing between different types of label in the autoradiogram, together with the fact that a small number of cells is labeled with tritium only, will make this measurement even more uncertain.

Our findings concerning the cell kinetic parameters may be viewed in the light of Potten's [2] description of the cellular organization of the epidermis as being made up of functional "epidermal proliferative units," which consist of a stem cell, a number of basal cells, and a column of mature cells leading to the cornified stratum that (in our case) is being shed. As evidenced by radiobiologic and autoradiographic studies on mouse epidermis, different proliferative subpopulations exist with different kinetic parameters. Potten likewise maintains that the degree of labeling is not the same for all cells.

An attempt has therefore been made to apply the results described above to a model resembling that proposed by Potten [2]. The assumptions are: (1) A stem cell divides to form 1 stem cell and 1 cell of first generation, which divides, leading to 2 second generation cells, then to 4 third generations cells. (2) The fourth generation cells do not divide but migrate in the

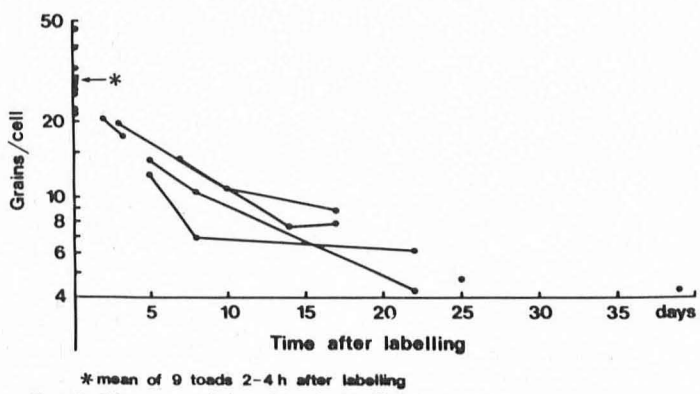

FIG 2. Mean number of grains/cell in stratum germinativum as function of time after injection of the DNA-precursor in a semi-log scale. Results from the same toad connected by *lines*. The grain halving time of about 4 days is estimated from the tangent to the curves at time zero.

course of cell cycle time T, so that at any given time, the same number of third and fourth generation cells are present in str germinativum. (3) All these dividing cells have the same cycle time T while the stem cell has 2T. The length of the S-phase is the same for all cells including the stem cell.

The resulting distribution according to generation of cells in str germinativum is shown in Table IV.

The system will then consist of 1 stem cell and $\frac{1}{2} \cdot (5 + 6) = 5\frac{1}{2}$ daughter cells. The labeling index was found to be 7.9% and hence the LI for stem cells (LI_{st}) and daugher cells (LI_d) can be calculated according to the following equation:

$$\frac{1}{13}[(1 + 2 + 4)\ LI_d + 2\ LI_{st}] = 0.079 \text{ and } LI_d = 2\ LI_{st}$$

Therefore,

$$\frac{1}{13}(7\ LI_d + 1\ LI_d) = 0.079$$

$$LI_d = \frac{0.079 \cdot 13}{8} = 0.128 \text{ and } LI_{st} = 0.064$$

Thus, the labeling index and the average degree of labeling of the cells at times 0, T, 2T, 3T, and 4T can be calculated, as shown in Table V.

It can be seen that the labeling index increases from 7.9% to 11.3% in the course of the first cycle and then decreases below the original value during the next few cycles. This trend is present in the experimental results shown in Fig 1.

The observations presented above support the assumption of a cell population in str germinativum of the toad skin which is inhomogeneous with respect to some cell kinetic parameters, and are compatible with Potten's model for the cellular structure of, e.g., mouse epithelium. However, Potten's model does not help to explain the observed overproduction of cells in the epidermis.

The authors gratefully acknowledge the valuable assistance of Miss Elise Frederiksen who succeeded in preparing excellent histologic sections of toad skin. Miss Frederiksen's careful observations and analyses of the autoradiograms form the basis of the present study.

TABLE III. *S-phase determinations by double labeling with thymidine**

A. One hour between first and second injection (8 animals)

Dose ratio	No. of cells counted	No. labeled with		S-phase (hr)
		^3H	^{14}C with/without ^3H	
10:1	554	6	57	9.5
10:1	400	5	38	7.6
10:1	420	3	12	4.0
10:1	462	3	18	6.0
10:1	436	3	25	8.3
10:1	486	3	23	7.7
12:1	403	6	26	4.3
12:1	450	6	52	8.7

B. Five hours between first and second injection (2 animals)

No. of cells counted		Percent labeled		S-phase (hr) (a)	No. of cells after two injections labeled with			S-phase (hr) (b)
		^3H	$^3H + {}^{14}C$					
Before Second	After injection	Before	After Second injection		^3H	$^3H + {}^{14}C$	^{14}C	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
556	514	9.0	7.4	6.1	8	38	12	6.3
835	415	14.3	7.7	9.3	32	32	13	8.5

* The S-phase determination is based on the ratio

$$\frac{\text{number of } (^{14}C\text{-labeled cells + double labeled cells})}{\text{number of cells labeled with } ^3H \text{ only}}$$

(a) $\frac{(3)}{(4)} \cdot 5$ hr

(b) $\frac{(6) + 2(7) + (8)}{2(7)} \cdot 5$ hr

TABLE IV. *Calculated averages for percent labeled cells and degree of labeling as a function of time**

Generation: relative abundance, %:		Stem cell 15%	First 8%	Second 15%	Third 31%	Fourth 31%	Average labeling	
							%	degree
Time								
0	Percent labeled	6.4	12.8	12.8	12.8	0.0	7.9	.
	Degree of labeling	1	1	1	1	1		1
		↘	↘	↘	↘			
1T	Percent labeled	6.4	6.4	12.8	12.8	12.8	11.3	.
	Degree of labeling	0.5	0.5	0.5	0.5	0.5		0.5
			↘	↘	↘			
2T	Percent labeled	6.4	0	6.4	12.8	12.8	9.9	.
	Degree of labeling	0.5	.	0.25	0.25	0.25		0.29
		↘		↘	↘			
3T	Percent labeled	6.4	6.4	0	6.4	12.8	7.4	.
	Degree of labeling	0.25	0.25	.	0.125	0.125		0.18
			↘		↘			
4T	Percent labeled	6.4	0	6.4	0	6.4	3.9	.
	Degree of labeling	0.25	.	0.125	.	0.0625		0.15

* Arrows indicate direction of division.

TABLE V. *Distribution of cells according to generation calculated on the basis of Potten's model (stratum germinativum)*

	Relative abundance	%
Stem cell	2	15
First generation	1	8
Second generation	2	15
Third generation	4	31
Fourth generation	4	31
	13	100

APPENDIX

Background Calculation

Background was determined on 40 different preparations and the average background was found to be 7.8 grains/3600 μm^2. In "thin" emulsions, the average was 7 grains/3600 μm^2, in "thick" emulsions, such as those used in connection with S-phase determinations, it was 10 grains/3600 μm^2. The grain distribution in different fields on the same preparation was in agreement with the Poisson distribution. On two preparations of inactive tissue coated with a thin emulsion layer, the number of grains/nucleus was counted over 293 and 447 cells, respectively. The results were 0.14 and 0.12 grains/nucleus—the distribution again being in agreement with the Poisson distribution—corresponding to an average surface area of the cell nuclei in our preparations of 72 μm^2 and 62 μm^2, respectively. Under the microscope was found an average of 40 μm^2 and a maximum of 65 μm^2.

Using the highest figure, we arrive at

$$\frac{14.2 \cdot 65}{3600} = 0.26$$

grains/nucleus which means, according to the Poisson distribution, that 0.15 out of 1000 nuclei carry 4 or more grains due to background. Hence, background does not affect our measurements of the LI, but does have some—however slight—effect on grain distributions, especially in the case of weakly labeled cell populations.

Estimate of the Biologic Mean and Variance of the Labeling Index

It is assumed that:

(1) the theoretical labeling index, λ, for a random toad is normally distributed with mean μ and variance σ^2.

(2) for a given toad, the observed labeling index x is binomially distributed with the parameters: number of cells counted, n, and labeling index, λ, for the given toad,

(3) the binomial distribution according to (2) is fairly well represented by the Poisson distribution with the mean $n\lambda$.

In the observed distribution of the labeling index from a random toad, the mean is called m_1, the variance m_2, and the cumulants κ_i.

The cumulants are determined by the expression

$$\exp\left\{\sum_{r=1}^{\infty} \kappa_r \cdot \frac{t^r}{r!}\right\} = \sum_{x=0}^{\infty} \exp\{tx\}$$

$$\cdot \int_{-\infty}^{\infty} \frac{(n\lambda)^x}{x!} \exp\{-n\lambda\} \cdot \frac{1}{\sqrt{2\pi\sigma^2}} \exp\left\{-\frac{1}{2}\frac{(\lambda-\mu)^2}{\sigma^2}\right\} d\lambda$$

where the right hand side can be reduced to

$$\exp\left\{t\mu n + \frac{t^2}{2}(n^2\sigma^2 + \mu n) + \cdots\right\}.$$

Because $\kappa_1 = m_1$ and $\kappa_2 = m_2$ we have

$$\kappa_1 = n\mu = m_1$$

$$\kappa_2 = n^2\sigma^2 + n\mu = m_2.$$

We have observed the labeling index for κ toads, where n_i denotes the number of counted cells for toad number i. From

$$m_1\{x_1 + x_2 + \cdots + x_k\} = \mu(n_1 + n_2 + \cdots + n_\kappa) \approx x_1 + x_2 + \cdots + x_\kappa$$

we arrive at the estimate

$$\mu \approx \frac{x_1 + x_2 + \cdots + x_\kappa}{n_1 + n_2 + \cdots + n_\kappa} = h.$$

From

$$m_2\{x_i\} = n_i^2\sigma^2 + n_i\mu \approx (x_i - n_i\mu)^2$$

we obtain by summing—there is stochastic independence between toads

$$\sigma^2 \cdot \sum_{i=1}^{k} n_i^2 + \mu \sum_{i=1}^{k} n_i \approx \sum_{i=1}^{k} (x_i - n_i\mu)^2$$

or the estimate

$$\sigma^2 \approx \frac{1}{\sum_{i=1}^{k} n_i^2}\left(\sum_{i=1}^{k}(x_i - n_i h)^2 - h\sum_{i=1}^{k} n_i\right).$$

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